STUDIES ON THE STABILITY OF THE CHOLINE OXIDASE

BY G. S. EADIE AND FREDERICK BERNHEIM

(From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina)

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The choline oxidase was first described by Bernheim and Bernheim (1). Later it was shown (2) that choline at pH 6.7 was oxidized to betaine aldehyde which was then only slowly oxidized further, whereas at pH 7.8 the oxidation proceeded rapidly to betaine. This indicated that betaine aldehyde was oxidized by another enzyme system, and Klein and Handler (3) were able to show that its oxidation required DPN but that of choline did not. The choline oxidase is inhibited by fatty acids (4) and its activity is greatly depressed in fatty livers (5). Its importance in the development of fatty livers caused by choline deficiency is shown by the fact that guinea pigs which lack the enzyme cannot be made to develop fatty livers (6) and hamsters which have some enzyme but much less than rats never accumulate as much liver fat on a choline-deficient diet as rats do (7). The enzyme can thus be considered a disadvantage when minimal amounts of choline are present for fat transport and metabolism. On the other hand, the recent work of Dubnoff (8) and Muntz (9) shows that it plays an important rôle in transmethylation reactions, for apparently only betaine aldehyde or betaine can act as a methyl donor. Animals which lack the enzyme can therefore presumably obtain their methyl groups from methionine only. Because of the importance of the enzyme a further study of its properties seems in order. It has previously been shown (1) that it is cyanide-sensitive and that it is readily inhibited by copper and phenylhydrazine (10). Barron and Singer (11) place it in the group of sulfhydryl enzymes. It is very sensitive to diamidines (12). The following is a study of the stability of the enzyme and the effect of cystine and certain metal ions upon it.

EXPERIMENTAL

10 to 12 gm. of rat liver were chopped with scissors, ground in a mortar with 10 to 15 ml. of 0.05 M K-Na-phosphate buffer of pH 6.7, and squeezed through muslin. The suspension was diluted to 50 ml. with water and centrifuged 10 minutes, and the supernatant discarded. 10 ml. more of buffer and 40 ml. more of water were added to the solid which was mixed

\footnote{Diphosphopyridine nucleotide.}
The resulting solid was mixed with 1.0 to 2.0 ml. of buffer and 0.5 ml. used in each Warburg vessel; the total fluid volume was 2.0 ml. Without added substrate the suspension took up little oxygen.

The rate curves thus obtained are complex. On the assumption that the oxidation of choline is a first order reaction, the aldehyde oxidation being disregarded for the moment, log \((a - x)\) was plotted against \(t\). The values chosen for \(a\) corresponded to the uptake of 1 atom of oxygen for each molecule of choline. The curves obtained in this way were of considerable diversity, but could be divided into three groups.

In the first group all points fell on a straight line; there is therefore no oxidation of betaine aldehyde in these experiments, and the oxidation of choline is a first order reaction. In the second group, although the points tended to fall on a curve, in many instances this was true only after 30 to 60 minutes or more, the earlier points falling on a straight line. This indicates that after an interval oxygen uptake became faster than expected. In these cases the total oxygen uptake exceeded the amount calculated on the basis of 1 atom of oxygen for each molecule of choline. The increased rate is therefore due to oxidation of the aldehyde, beginning somewhat later. It has been unfortunately impossible to analyze the data for the second reaction, but in general it apparently tends to begin earlier when the velocity constant of the first reaction is greater, i.e. at low substrate concentrations. The reason for the delay is unknown.

In the third group the points fell on a curve convex to the left, and in no case was there an initial linear phase. In these experiments oxidation ceased before 1 atom of oxygen was taken up per molecule of choline, although there was no constant end-point. Inactivation of enzyme was the probable cause of the deviation, and this was shown to be the case, both in the presence and absence of substrate. The effect was twice as great at pH 6.7 as at pH 7.8. Thus, if the enzyme suspension was incubated 45 minutes at 37° before the addition of choline, the rate of oxidation was about half the original at pH 6.7 and about three-fourths at pH 7.8. Because the loss of activity was greatest initially, the assumption was made that the inactivation of enzyme was also a first order reaction, i.e. \(\frac{dE}{dt} = cE\), where \(E\) is concentration of active enzyme at \(t\) minutes and \(c\) is a constant characterizing the rate of inactivation. On integration this gives \(E = E_0e^{-ct}\), \(E_0\) being the concentration of active enzyme at zero time. On the additional assumption that the rate of oxidation of choline is proportional to the concentration of active enzyme, the rate equation becomes \(\frac{dx}{dt} = -kE(a - x)\). On substituting for \(E\) and integrating, this becomes

\[
\log \frac{a}{a - x} = \frac{k}{c} E_0(e^{-ct} - 1) \tag{1}
\]
Accordingly if \( \log (a - x) \) is plotted against \( (e^{-ct} - 1) \), the points should fall on a straight line, and this was found to be the case (Fig. 1). It was possible to change every curve of the third group into a straight line by choosing appropriate values for \( c \) and plotting in this way. The slope, \( (k/c)E_0 \), for each of these lines was calculated by the method of least squares; its value multiplied by \( c \) gave \( kE_0 \).

To determine the rate of inactivation of the enzyme in the absence of substrate, the enzyme preparation was placed in the Warburg vessels at zero time and the substrate added at \( t_1 \) minutes. Assuming that the enzyme concentration has decreased to \( E_1 \) at \( t_1 \) minutes, equation (1) becomes

\[
\log \frac{a}{a - x} = \frac{k}{c_1} E_1 e^{-c_1(t - t_1)} - 1
\]

\( c_1 \) being the constant of the rate of inactivation in the presence of substrate. \( kE_1 \) is calculated as before. If inactivation follows the same rule in the absence of substrate, the concentration of active enzyme when the substrate is added will be given by \( E_1 = E_0 e^{-c_2 t_1} \) or \( \ln kE_1 = kE_0 - c_2 t_1 \), \( c_2 \) being the inactivation constant in the absence of substrate. If several determinations of \( kE \) are made at different times, their logarithms plotted against \( t \) should fall on a straight line. This was found to be the case (Fig. 2). The slope of the line, 0.018, is equal to \( c_2 \), the constant of inactivation in the absence of substrate.

Because inactivation is a first order reaction, either the enzyme is in itself a very unstable molecule, or inactivation results from a reaction with
some substance present in sufficiently great amount so that its concentra-
tion is not significantly altered by any combination with enzyme. Inac-
tivation occurred whether the vessels were shaken or not, which indicates
that surface denaturation of the enzyme is not the cause. It also occurred
equally well at oxygen tensions of 2 per cent and in the presence of suffi-
cient cyanide to inhibit the oxidation of choline about 30 per cent. The
presence of substrate, however, decreased the rate of inactivation; in the
experiment cited above, for example, $c_1$ (presence of choline) varied from
0.004 to 0.010 with different substrate concentrations, while $c_2$ (absence of
choline) was 0.018.

Cystine was found to accelerate inactivation very markedly at pH 6.7,
less at pH 7.8. The effect of L-cystine in the presence of choline is shown
in Fig. 3; in the absence of choline the rate of inactivation was consider-
ably greater. Oxidized glutathione in equimolecular concentrations did
not affect the rate of inactivation. It was assumed that cystine oxidized
the $-\text{SH}$ groups on the enzyme and that it or a similar substance in the
preparation accounted for the "spontaneous" inactivation which would
therefore be independent of the oxygen pressure. The effect is, however,
fairly specific. In the first place, copper ions which are a general catalyst
for $-\text{SH}$ groups do not catalyze the inactivation of the choline oxidase as
cystine does. \( 1 \times 10^{-4} \) m copper sulfate when added with choline to the enzyme caused an 18 per cent inhibition; if added 30 minutes before the choline, the inhibition was 19 per cent; substrate therefore does not protect. The corresponding figures on the same preparation for \( 0.83 \times 10^{-3} \) m cystine were 20 and 63 per cent, showing marked protection. Secondly, the succinoxidase which also contains \(-SH\) groups is not inactivated by incubation with or without cystine under similar conditions, although it is readily inactivated by copper. The effect of incubation with cystine on the choline and succinoxidase at similar rates of oxidation is shown in Table I. Finally, \( DL\)-cystine was as effective in accelerating inactivation as an equimolecular amount of \( L\)-cystine. In one experiment the value of

\[ kE_1 \]

for enzyme shaken 30 minutes in the presence of 0.4 mg. of \( L\)-cystine was 0.0051; with the same amount of \( DL\)-cystine it was 0.0055. In the absence of cystine the destruction was less, \( kE_1 \) being 0.008. Both forms of cystine are as effective in crude liver suspensions as in the partially purified preparation.

In an attempt to block the second oxidation, that of the aldehyde, semicarbazide was added. This also had the effect of increasing the rate of inactivation of the enzyme both in the presence and the absence of substrate (Fig. 4); the rate of inactivation was again greater in the absence of choline (\( c \) constants 0.032 and 0.0012 per mg. of semicarbazide respectively).

It seemed probable that nickel, cobalt, and iron salts would also react with \(-SH\) groups, partly as catalysts of their oxidation, but also by form-
ing complexes with the enzyme. In order to investigate this, the metals as sulfates were dissolved in water and added to the liver suspension in phosphate buffer. The effect on the rate of inactivation is shown by an experiment in which the constant in the presence of choline was found to be 0.001; on the addition of 22 γ of cobalt it rose to 0.012. Further addition of cobalt had little effect on this value. In the second place the metallic ions inhibit the enzyme. To determine whether this inhibition is competitive or not, the Michaelis constant was estimated by determining the values for $kE_0$ (equation (1)) for three or four different concentrations of choline. Five such experiments were carried out and the values of $K_m$ on successive pooling of data were 0.0022, 0.0012, 0.0015, 0.0016, and 0.0017, with 13 degrees of freedom when values are expressed in moles, liters, and minutes. The $F$ test showed that these differences were not significant. On the addition of 22 γ of nickel to the reaction mixture, successive pooling gave 0.0096, 0.0088, and 0.0100 for the value of the slope, that is for $K_m(1 + (i/K_i))$, where $i$ refers to the molar concentration of inhibitor and $K_i$ is the inhibitor-enzyme dissociation constant. There were 7 degrees of freedom and the $F$ test showed that the variation in different determinations was not significant. The difference between this figure and the value for $K_m$ is sufficiently great to be beyond the range of experimental error and indicates that this is a case of competitive inhibition. The number of atoms of metal combining with 1 molecule (or active center) of enzyme is determined by plotting the reciprocal of the

### Table I

*Effect of 30 Minute Incubation with and without $0.88 \times 10^{-8} \mu$ Cystine at 37° on Activity of Choline and Succinoxidases*

<table>
<thead>
<tr>
<th>Min.</th>
<th>No incubation</th>
<th>30 min. incubation</th>
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<tbody>
<tr>
<td></td>
<td>Choline</td>
<td>Succinate</td>
</tr>
<tr>
<td></td>
<td>Choline + cystine</td>
<td>Succinate + cystine</td>
</tr>
<tr>
<td>10</td>
<td>28</td>
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0.5 ml. of liver suspension was used for the choline oxidase, 0.1 ml. for the succinoxidase. 2.0 mg. of each substrate were used. The figures are in c.mm. of O_2 uptake. The appropriate control uptakes have been subtracted.
initial velocity against the concentration of inhibitor. In every experiment with all three metals a straight line was obtained when the first power of the inhibitor concentration was used, which indicates that 1 atom of the inhibitor combines with 1 unit of enzyme. The value for $K_i$, from the last figure of each series, was found to be $0.38 \times 10^{-4}$. On addition of 42 $\gamma$ of cobalt successive poolings gave 0.0052, 0.0066, 0.0076, and 0.0074 for the value of the slope. There were 11 degrees of freedom and the $F$ test was not significant. The straight line obtained on plotting the reciprocal of the initial velocity against the first power of the inhibitor concentration is shown in Fig. 5. From these figures $K_i$ was found to be $1.1 \times 10^{-4}$. On the addition of 37 $\gamma$ of iron, the successive pooled values of the slope were 0.014, 0.017, and 0.017 with 9 degrees of freedom. Again no significant variation was found by the $F$ test, and the relationship between $1/v$ and the inhibitor concentration was linear. $K_i$ was found to be $0.37 \times 10^{-4}$.

These salts are thus effective competitive inhibitors of the choline oxidase, but under comparable conditions have no effect on the succinoxidase, even when the suspension is diluted so that succinic acid and choline are oxidized at the same rates. Other enzymes in the washed suspension, such as L-proline oxidase, amine oxidase, and D-amino acid oxidase, are also unaffected by this concentration of the metals. Manganese in 4 times the concentration does not inhibit the choline oxidase. The af-
finity of the metallic cations for the active groups of the liver protein is high enough, as shown by the dissociation constant, so that insoluble phosphates are not formed to an appreciable extent. This is confirmed by the fact that, if the phosphate concentration is lowered, the inhibition of the enzyme is not altered. Additional evidence is summarized in Fig. 6. When 8-hydroxyquinoline is added before the nickel, no inhibition occurs. If it is added 30 minutes after the nickel, the inhibition is only very slowly decreased, which indicates a very small rate of dissociation of the nickel enzyme complex.

![Graph](http://www.jbc.org/)

**Fig. 6.** The effect of 8-hydroxyquinoline added before and 30 minutes after 0.1 mg. of NiSO₄ on the oxidation of choline. Curve A, control, with 0.5 mg. of 8-hydroxyquinoline; Curve B, 8-hydroxyquinoline added before the nickel; Curve C, 8-hydroxyquinoline added 30 minutes after the nickel; Curve D, nickel alone.

**SUMMARY**

1. Choline oxidase partially purified rapidly loses its activity. The rate of loss is that of a first order reaction and equations are derived to describe the rate of reaction as modified by this. The inactivation is more rapid at pH 6.7 than at 7.8.

2. Cystine and semicarbazide increase the rate of inactivation both in the presence and absence of choline.

3. Nickel, cobalt, and iron salts not only increase the rate of inactivation, but also combine reversibly with a group on the enzyme to inhibit it. Manganese salts have no effect in comparable concentrations.

4. The specificity of these effects is described.
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